IDENTIFICATION OF AUTOANTIGENS IN AUTOIMMUNE ENDOCRINE DISEASES

By

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A dedication to my parents and my husband:

for their love

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Abstract of Dissertation Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

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Type I autoimmune polyglandular syndrome (APS) consists of three primary disorders: Addison's disease, chronic mucocutaneous candidiasis and hypoparathyroidism as well as several commonly associated disorders such as gonadal failure, alopecia and vitiligo. The search for specific targeted autoantigens involved in APS has been the major focus of my thesis.

Human parathyroid gland was homogenized and cytosolic and membrane fractions were obtained by ultracentrifugation. Specific autoantigens with molecular weight of 70 and 80 kDa were found in the cytosolic fraction and a 120-140 kDa autoantigen was localized to the membrane fraction. The 120-140 kDa autoantigen has the same molecular weight as the calcium sensing receptor (Ca-SR), therefore, the Ca-SR was investigated as a candidate autoantigen. Patient sera were

tested in parallel with a rabbit anti-Ca-SR antibody in immunoblots using a Ca-SR-transfected HEK-293 cell membrane as the antigen source. Autoantibodies from patient sera reacted to a 120-140 kDa protein which was identical in molecular weight to that recognized by the rabbit positive control antibody. The Ca-SR was then translated into extracellular and intracellular domains by an *in vitro* rabbit reticulocyte system. Autoantibodies recognized only the extracellular domain. Therefore, the extracellular domain of Ca-SR has been identified as the major autoantigen in hypoparathyroidism.

The sperm receptor ZP3 has been identified as an autoantigen in experimental murine autoimmune oophoritis. In this study, the human ZP3 cDNA was translated *in vitro* and immunoprecipitated with a monkey anti-ZP3 antibody and sera from patients with gonadal failure. The monkey antibody was the only one which reacted with ZP3. Therefore, autoantibodies against ZP3 were not found in human patients. Autoantigens involved in alopecia were investigated using human head skin homogenate as antigen source. Specific autoantigens with molecular weights of 46-55, 57 as well as muultiple proteins with molecular weight higher than 100 kDa were identified by immunoblot.

The identification of the autoantigens involved in APS is an important step toward the understanding of the pathogenic mechanisms of the diseases and it may also lead to the

development of specific methods for diagnosis as well as prevention of the diseases.

CHAPTER 1
INTRODUCTION

Autoimmune Diseases

Overview

One of the most important features of the immune system is its ability to distinguish foreign antigens from self antigens. Normally, each individual's lymphocytes are able to recognize and attack foreign invaders, but remain unresponsive to self antigens. The condition of unresponsiveness to self antigens is called immunological tolerance. The breakdown of tolerance to self antigens presumably leads to initiation of an autoimmune process which leads to autoimmune diseases.[1-3]

Autoimmune diseases are estimated to affect as many as 5 to 7 percent of adults in Europe and North America. In the United State alone, 1 to 2 percent of the entire population may be affected. This poses a serious women's health issue in that two-thirds of all patients with an autoimmune disease are women. If autoimmunity can be shown to play a major role in atherosclerosis, the leading cause of deaths in the Caucasion population, the ratio of the affected population would be much

further increased.[2,4]

Historical evidence suggests that autoimmune diseases became more common in the 19th century, although this could be an artifact of increased recognition.[5] Most autoimmune diseases are polygenic, whose expression reflects the interaction between both genetic and environmental factors which contribute to the development of these diseases.[1,6]

Classification of Autoimmune Disorders

Autoimmune diseases can generally be placed into two categories: organ-specific and non-organ-specific autoimmune disease. In organ-specific autoimmune diseases, specific organs or tissues are affected. Insulin dependent diabetes (IDD), Addison's disease and Graves' disease are examples of diseases in this category.[7] Alternatively, non-organ-specific autoimmune diseases usually involve multiple organs or tissues. Systemic lupus erythematous (SLE) and rheumatoid arthritis are in this category.[8] The autoantigens in organ-specific autoimmune diseases are often unique to the targeted tissues, and include tissue specific membrane receptors, enzymes and secreted hormones (Table 1).[9] The autoantigens in non-organ-specific disorders are usually found ubiquitous in all parts of the body include collagen, DNA and histone.[10,11]

Table 1. The classification of autoimmune diseases.

Organ-specific Autoimmune Diseases	Organ/cell	Target Antigen
Addison's Disease	Adrenal cortex	21-hydroxylase
Autoimmune hemolytic anemia	Red blood cell membrane protein	
Autoimmune Hypoparathyroidism	Parathyroid Gland	Cacium-sensing receptor
Chronic active hepatitis	Hepatocyte	P450IID6
Graves' disease	Thyroid	TSH receptor
Hashimoto disease	Thyroid	T3,T4,TSH and TPO
Hypogonadism	Leydig cells Testes/Ovary	P450scc and 17α-hydroxylase
Insulin dependent diabetes	Pancreatic islet cells	GAD65 and GAD67, tyrosine phosphatase
Multiple sclerosis	Brain and spinal cord	Myelin basic protein
Myasthenia gravis	Nerve/muscle synapses	ACTH receptor
Pernicious anemia	Gastric parietal cell	H,K-ATPase and intrinsic factor
Vitiligo	Melanocyte	Tyrosinase
Non-organ Specific Autoimmune Diseases		
Rheumatoid arthritis	Connective tissue	Type II collogen
Systemic lupus erythematosus	Kidney, skin, platelet	DNA,histone

Genetic Factors in Autoimmunity

Based on the rates of familial transmission, genetic predisposition is an important factor in the development of autoimmune diseases.[6] It is now believed that multiple genes may contribute to the induction of autoimmunity.[1] The major histocompatibility complex (MHC) genes are associated with most autoimmune diseases such as IDD.[12-14] The probable reason for this correlation is that MHC molecules play key roles in antigen presentation, T cell selection and activation.[1] Individuals who carry particular HLA haplotypes such as DRB1*03/DQB1*0201 and/or DRB1*04/DQB1*0302 are more likely to develop type I diabetes. However, not all individuals develop autoimmune diseases even though they carry disease-associated HLA alleles.[15] Therefore, a particular HLA allele itself is not sufficient to cause autoimmune diseases. A triggering factor such as viral infection or other environmental factors appears also to be required to initiate or induce the development of the autoimmune process.[4]

In addition to the research on the role of MHC, some research groups have focused on T cell receptor (TCR) genes, since T cells play a major role in autoimmune process. Even though a few studies have shown that TCR genes contributed to autoimmune diseases,[16-18] no strong evidence supporting the association between specific TCR haplotypes and autoimmune

disorders has yet been clearly proven.[19]

Etiological Factors in Autoimmunity

1. Under normal conditions, proteins of certain tissue such as the lens of the eye are concealed from the immune system. However, once the tissues become damaged, the released proteins may provoke an autoimmune response because tolerance to them had not been established.

2. Hormonal influences are also important in certain autoimmune diseases. Females are much more prone to hormone influence than males, and tend to have much higher rates of autoimmune diseases.[4]

Howard S.Fox of Scripps has shown that the female hormone estrogen can stimulate the transcription of gamma-interferon (IFN-γ). IFN-γ is a cytokine that can increase the expression of MHC class II by antigen presenting cells, induce the production of other cytokines produced by TH1 cells and activate macrophages.[20] Thus, estrogen could influence the autoimmune process by increasing the production of IFN-γ.[21]

3. Viral and bacterial infection may be associated with autoimmunity.[22-23] A good example of this is rheumatic fever in which the inflammation of the heart and joints follow a streptococcal pharyngitis. The autoimmune lesions in the heart and its valves are not due to the bacteria itself but rather

due to the host's immune responses to it.

It should be noted that microorganisms may induce autoimmune diseases not only through molecular mimicry,[24-26] but also through other means such as the release of superantigens,[27-29] shift in the spectrum of cytokine production[30] and the release of sequestered antigens.[31-34]

Mechanisms of Autoimmunity

Since class II MHC-restricted helper T cells play a central role in all acquired cellular and humoral immune responses, while central T cell clonal deletion or peripheral anergy are normally effective ways of maintaining tolerance to self antigens, it is believed that autoreactive T cells play major roles in the autoimmune process.[35-40] Even in the known autoantibody-mediated disorders, the defect depends on the appearance of autoreactive TH2 lymphocytes, which are required for the production of high-affinity autoantibodies.[41]

Autoreactive T Cells

Since the variable genes encoding T cell receptors that may recognize self antigens are present in the germ line, all individuals have the potential to develop an autoimmune reaction. Therefore, mechanisms to prevent the maturation of

autoreactive T cells are necessary. Fortunately, there are such mechanisms present in normal individuals.[42,43] Autoreactive T cells to protein antigens that are present in the thymus are deleted by negative selection.[44-46] T cell tolerance to protein antigens that are not present in the thymus is induced in the periphery.[47-51]

Autoimmunity may develop if autoreactive T cells escape the negative selection process and are allowed to mature and leave the thymus.[52] Autoimmunity may also develop if peripheral tolerance fails and inadequately anergized T cells are activated by polyclonal activators, sequstered antigens, or by cross-reaction between self antigens and infectious pathogens.[53,54]

1. Failure of self-tolerance in thymus.

The process which eliminates or inactivates potentially autoreactive T cell clones in the thymus is called negative selection.[55-57]

The elimination of autoreactive T lymphocytes in the thymus is induced by the expression of self antigen by the thymic epithelium. If immature T cells in the thymus bind antigen via either class I or class II MHC molecules with high affinity, the immature T cells are killed by apoptosis or rendered functionally inactive.[58,59] Autoreactive $CD8^+$ T cells are eliminated via class I dependent antigen presentation and $CD4^+$ T cells via class II dependent antigen presentation.

Functional inactivation in the thymus also can be induced by the lack of a second signal on the antigen presenting cells, or by insufficient levels of antigens present in the thymus.[43,60] In order to have negative selection take place, the self-antigen must be present in sufficient quantity in the thymus. It is possible that some circulating macrophages collect cell debris, process and present these materials in the thymus, even though they are not actually created there.

Self-tolerance mechanisms in the thymus may be disrupted in many different ways. First, the expression of particular MHC alleles may influence clonal deletion of autoreactive T lymphocytes by the strength of the affinity of peptide associated with the MHC binding groove.[59] Second, some sequestered self-antigens may not reach the immune system/thymus during the development of tolerance which occurs during late fetal or early neonatal life in mammals. Third, defects in apoptosis genes (such as Fas or Fas ligand genes) may lead to inadequate clonal deletion. It has been shown that the autoreactive thymocytes escape from apoptosis in the thymus, then go to the periphery in lpr mice.[61] Other studies argue against the role for Fas in clonal deletion in the thymus, but support a role of Fas-mediated apoptosis in peripheral tolerance.[62] Fourth, the concentration of self peptides in the thymus may play a role in clonal deletion. It has been suggested that any peptide that is present

continuously in the thymus at a concentration greater than 10μM reliably deletes reactive T cells unless for some reasons the mechanism is defective.[63]

2. Failure of self-tolerance in periphery.

It is unlikely that all self antigens can be presented in the thymus, therefore, alternative mechanisms are required to render autoreactive T lymphocytes which have escaped thymic negative selection unresponsive. Clonal anergy is the major process for inducing self-tolerance in periphery, especially for antigens present only in peripheral tissues and not in the thymus. If T cells recognize an MHC-associated antigen on antigen presenting cells (APCs) that do not present the necessary costimulators (eg, B7) for T cell activation, the T cells are placed into a stage of anergy where they can become unresponsive to subsequent stimulation by the same antigen in the presence of costimulators.[64,65] The interaction between B7.1 and CD28 molecules is required for T cell activation. It has been shown that APCs lacking B7 on their surfaces can induce T cell anergy.

Self tolerance in periphery can also be disrupted by several immunological mechanisms.

(1). Sequestered antigen.

Some sequestered antigens may be expressed as apparently "new" antigens as the result of trauma. Alternatively, some self antigens may be partially degraded and lead to "new"

(i.e, cryptic) antigenic targets for the immune system. Autoimmune disease may develop if the new antigens are recognized as foreign.[34]

(2). Molecular mimicry.

Peripheral self-tolerance can also be disrupted through the concept of molecular mimicry.[24,66] Molecular mimicry is defined by a structural homology in a linear amino acid sequence between self and invading pathogen (e.g., viral or bacteria proteins). This situation is primarily applicable to peptide-specific Th1 cells and cytotoxic T cells (CTLs) rather than to antibodies, since the latter tend to recognize conformational determinants. However, antibody reactivities to linear peptides shared by self and foreign molecules have also been reported.

(3). Polyclonal activation.

Polyclonal activation refers to T cell activation by superantigens. Antigens that bind to all TCRs utilizing a specific Vß gene segment, regardless of the Vα gene segment, have been called superantigens. Superantigens stimulate a large percentage of all T cell clones at one time. In this process, autoreactive T cells which escaped the negative selection but are held in an anergized state in periphery, may be activated by such means.[27,28] They may proliferate upon subsequent encounter to a self antigen, and lead to an autoimmune process. A recent study from Schiffenbauer et al.[29]

has shown that the superantigens staphylococcal enterotoxins (SEs) B and A are able to induce relapse of EAE in PL/J mice by reactivating the autoreactive T cells. The authors also proposed an important hypothesis that autoimmune disease is induced by a "two-hit" process, i.e, the autoreactive T cells are first stimulated through a molecular mimicry mechanism after an infection. Autoimmune disease may not develop at this stage due to either insufficient numbers of autoreactive T cells or a supression mechanism. However, the clinical manifestation of the autoimmune disease will develop after a second infection with a superantigen-producing organism.

Autoreactive B Cells

Certain autoantigens are formed by polysaccharides and lipids, but these kinds of antigens are not recognized by MHC-restricted T cells. B cell tolerance may be the principal mechanism for unresponsiveness to such self antigens.[67] Compared to T cell tolerance, B cell tolerance is more difficult to induce and requires more antigen.[68,69]

There are two principle mechanisms of B cell tolerance induction: clonal deletion and clonal anergy.[70-72]

Clonal deletion may be induced when B cells mature in the bone marrow and encounter self antigens,[73] perhaps at the stage when they express only the IgM form of membrane

receptors for antigens. Using double-transgenic mice, Christopher C. Goodnow et al.[67] showed that B cell tolerance to lysozyme was due to functional inactivation or clonal anergy, rather than clonal deletion of self-reactive B cells. This finding suggests that clonal deletion plays a minor role in B cell tolerance, however this termination could be antigen dependent.

Clonal anergy may be induced by an antigen-receptor interaction or by lack of help from T cells, which can block the expression of membrane Ig before B cells mature to a stage of functional competence.[74] The net result of clonal anergy is that autoreactive B cell are often present in normal individuals but are functionally unresponsive to antigenic stimulation.

The failure of B cell tolerance may be explained by several hypotheses. First, anergized B cells can be induced to secrete autoantibodies by polyclonal activators, such as lipopolysaccharide (LPS) or Epstein-Barr virus (EBV), which function independently of membrane Ig.[75,76] Polyclonal B cell activation has been considered to be a contributing or initiating mechanism of autoimmunity, particularly in systemic autoimmune disease, such as SLE.[77-79] Second, the activation of self-antigen specific T cells which have escaped clonal deletion in the thymus can stimulate self-antigen specific B cells which are normally present but unresponsive to these

antigens since they lack T cell help. In this case, the autoantibodies may be specific to one or a few related antigens, and may lead to organ-specific autoimmune diseases.

The Role of Autoantibodies

In autoimmune disease, autoantibodies may be the agents causing the disease, the consequence of tissue damage, or simply indicators of an autoimmune process. The role of autoantibodies in the pathogenesis varies with the location of the targeted antigen.[80]

(1) Autoantibodies directed against cell surface targets, such as hormone receptors, play a major role in autoimmune disorders. One example is that anti-acetylcholine (Ach) receptor in myasthenia gravis (MS).[81-84] Another example is that anti-thyroid stimulating hormone (TSH) receptor in Graves' disease.[85,86]

(2) Those autoantibodies directed against extracellular targets, such as circulating hormones or extracellular matrix, may or may not cause any damage.[80]

(3) Those apparently directed against intracellular targets are usually not pathogenic.[80] However, the autoantibodies against DNA and histone are pathogenic in SLE.

While a few types of autoantibodies appear to be involved in the actual pathogenesis of autoimmune disease,

autoantibodies arising to self antigens provide important hallmarks of autoimmune diseases affecting specific organs or tissues. Autoantibodies can usually be detected long before the clinical onset of their associated diseases. The detection of the autoantibodies can therefore be useful for specific clinical diagnostic purposes, as well as for prediction and thereby for possible prevention of disease. In general, the detection of autoantibodies can be used to support an autoimmune etiology and to screen individuals at risk of developing autoimmune disease before their onsets of clinical syndrome. In pregnant women, the detection of certain autoantibodies may predict disease in their fetuses and newborns.

Methods to Detect Autoantibodies

The most commonly used methods to detect autoantibodies include indirect immunofluorescence,[87] enzyme-linked immunosorbent assay (ELISA),[88] radioimmunoassay (RIA),[89] western blot, and immunoprecipitation.[90,91]

1. Indirect immunofluorescence.

Patients sera that contain putative autoantibodies are allowed to incubate with a frozen section of the target tissue, e.g., pancreas, and the residual sera are then washed away with PBS (phosphate-buffered saline). The binding of

autoantibodies to the section can be detected by the subsequent incubation of a secondary antibody labelled with a fluorescent dye such as FITC. After washing, the stained antigens are visualized by a fluorescence microscope equipped with a ultra violet light source. This technique is very sensitive and can provide information on the localization of the antigens within a tissue.

Indirect immunofluorescence is currently being used as a routine tool to detect autoantibodies against antigens in various tissues such as adrenal cortex, gonad, pancreatic islet, gastric mucosa, and thyroid. The detection of these autoantibodies has been very useful in the diagnosis of organ-specific autoimmune diseases such as Addison's disease, hypogonadism, IDD, pernicious anemia and autoimmune thyroid diseases.

2. ELISA.

The antigen used in this method must be soluble and able to be coated onto the wells of a 96-well microtiter plate. Bovine serum albumin is then added to the wells to block non-specific binding sites. Diluted patient sera are added and incubated with the antigen. After washing, a secondary antibody conjugated with an enzyme is added. After washing again, the binding of the autoantibodies to antigens is visualized by adding a soluble substrate to develop a color reaction. The titer of autoantibodies is determined by

spectrophotometer and the intensity of the color is proportional to the concentration of autoantibodies. ELISA is an extremely rapid technique which is relatively inexpensive, therefore, it has been used for initial screening of large number of samples. However, some conformation-dependent antigenic epitopes may be lost upon the binding of the antigen to the plastic wells and autoantibodies to such antigens will not be detected.

3. Western blot.

The technique of transferring proteins from gel to membrane and the subsequent incubation with an antibody is called Western or immunoblotting. This technique has been used extensively in the detection of tissue autoantigens that react to autoantibodies by their linear epitopes. The tissue autoantigen has to be solubilized first and then subjected to size separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins are then transferred onto a membrane such as Immobilon-P or nitrocellulose. Diluted patient sera are then incubated with this membrane. Autoantibodies affixed to the antigen-containing membrane are detected by the addition of a secondary antibody such as a goat-anti-human IgG coupled with an enzyme such as alkaline phosphatase. Water-insoluble substrate is then added to the membrane to visualize the specific antigens recognized by the autoantibodies.

Immunoblotting is often performed after an autoantigen has been demonstrated by immunofluorescence. This technique allows the detection of specific molecular weight antigenic proteins. Knowledge of the molecular weight of the antigen will assist in further characterization of the antigen. Since immunoblot usually involves the separation of protein under denaturing conditions, it would avoid the problems of solubilization, aggregation, and co-precipitation of other proteins. However, conformational epitopes may not be detected by this method. Polyclonal antisera such sera from patients usually give higher background than monoclonal antibodies. This problem can be solved by including normal control sera in parallel to the patient sera in the blot.

4. RIA.

The technique was developed by Yalow and Berson in the late 1950s. After years of development, the methodology is now fairly simple and rapid. This technique requires the use of highly purified antigen. The antigen is usually labelled by ^{125}I and an aliquot of the labelled antigen is incubated with patients sera. The antibody-antigen complex is precipitated by adding protein A-Sepharose. After washing the unbound antigen, the concentration of the autoantibodies can be quantitated by counting the radioactivity of the antibody-antigen complex in a scintillation counter.

5. Immunoprecipitation.

This technique is used to isolate the autoantigen in mixture by using an autoantibody directed against this autoantigen. Sera are added to ^{35}S-labeled autoantigen. After incubation, protein A-sepharose is added and centrifugation is performed. This permits separation of the autoantibody-bound autoantigen which is present in the pellet and the unbound autoantigen which stays in the supernatant. The labelled antigen is then separated from the bound antibody by SDS-PAGE and visualized by autoradiography. This detection documents that the respective antibody must have been present in the patient sera.

In Vitro Translation

In vitro translation is a technique to express cloned genes in a cell-free system. Currently, rabbit reticulocyte lysate or the wheat germ system is being used.[92,93] In each of these systems, mRNA is translated into protein in the presence of a radioactively labeled amino acid such as ^{35}S-labelled methionine. The translated protein is usually in its native form and suitable for immunoprecipitation. The wheat germ system has been shown to be better suited for the translation of prokaryotic and plant mRNAs, whereas the reticulocyte system has been used for all other eukaryotic mRNAs.

Endogenous mRNAs have been eliminated in both systems by incubation with calcium activated nuclease. Therefore, background proteins are minimal in these systems.

In vitro translation can be used to identify translational products of mRNAs, to determine the translational efficiency of a particular mRNA and to study the mechanisms of protein synthesis.

Canine pancreas microsomal membranes are usually used to study cotranslational processing of proteins such as signal peptide cleavage, membrane insertion, translocation and glycosylation.

Autoimmune Polyglandular Syndromes(APS)

The APSs are characterized by the simultaneous occurrence of multiple autoimmune endocrinopathies and skin disease. APS can be classified into at least two groups based on the patients' ages of onset, and their associations with specific endocrine disorders and HLA phenotypes.[94,95]

The autoimmune nature of these diseases has been determined based on the presence of lymphocytic infiltration in the affected gland, organ specific autoantibodies in the serum, cellular immune defects and associations with HLA genes.[96] In contrast to systemic autoimmune diseases, the autoantigens of the polyendocrinopathies are organ specific.

Therefore, APS and systemic autoimmune diseases have distinct clinical features that rarely overlap.

The pathogenic role of autoantibodies against certain cellular receptors is well established, whereas those against intracellular enzymes are not. Many of the autoimmune endocrinopathies appear to be directly mediated by autoreactive CD8 cytotoxic T cells (CTL) with help from Th1 CD4 cells, while autoantibodies to cellular receptors which arise as part of a Th2 response may directly induce others. Mixed cellular and antibody autoimmune responses in any single disease are probably pathogenic to some degree.[9]

It is not known why the individual components of the APS cluster together. One possible explanation is that multiple and interactive genetic defects affect immunological tolerance. Another possible explanation is that endocrine glands may share common antigenic determinants such that autoimmunity against a single antigen must result in a process associated with involvement of other glands.

As an autoimmune endocrinopathy progresses, some immunological markers may disappear while others may appear. Autoantibody markers may also disappear due to elimination of the autoantigens which drive the response.

Type I APS

APS I is defined by the occurrence of at least two of the three diseases: chronic mucocutaneous candidiasis, acquired hypoparathyroidism, and autoimmune (autoantibody-positive) Addison's disease. These diseases tend to appear in the order of mucocutaneous candidiasis, acquired hypoparathyroidism, and Addison's disease.[97-99] If one of these three disorders is bypassed, it may not ever develop. Acquired hypoparathyroidism is the most common endocrinologic manifestation of type I APS, occurring in more than 80% of patients.

Type I APS is a relatively rare syndrome and usually presents early in infancy. Males and females are equally affected. APS I occurs either as an autosomal recessive disease, or as a sporadic disorder. APS I is not linked to genes within the HLA-DR genetic region of chromosome 6,[100] the responsible gene has been recently mapped to chromosome 21q22.3.[101]

Type I APS is also associated with other autoimmune diseases, such as early onset pernicious anemia, chronic active hepatitis, alopecia, vitiligo, malabsorption syndromes and gonadal failure (Table 2). Pernicious anemia and hypogonadism tend to occur last. Pernicious anemia occurs especially among older patients. Hypogonadism affects 50% of females but is diagnosed less commonly among males with type

Table 2. Characteristics of autoimmune disorders in APS I.

Disease	Mean Age of Onset	Extreme Ages
Acquired Hypo-parathyroidism	7.5 yr	3 mo - 22 yr (between 2 yr and 10 yr in 80%)
Mucocutaneous Candidiasis	5.5 yr	2 mo - 21 yr (between 2 yr and 7 yr in 80%)
Addison's Disease	13 yr	1.5 yr - 34yr (between 5 yr and 16 yr in 80%)
Gonadal Failure	at puberty	4 yr - 21 yr
Malabsorption	8 yr	1.5 yr - 30yr
Alopecia	8 yr	1 yr - 21 yr
Pernicious Anemia	16 yr	7 yr - 21 yr
Active Hepatitis	17 yr	5 yr - 21 yr
Thyroiditis	17 yr	15 yr - 18 yr
Vitiligo		3 yr - 28 yr
IDD		6 yr - 24 yr

I APS.

Alopecia and vitiligo may be seen in both APSs, and often the degree of these skin lesions is striking. Alopecia universalis where there is eventually an absence of all body hair is most often seen with type I APS. Difficulties with malabsorption are common in APS I, resulting from a variety of causes. Chronic active hepatitis is common among type I APS patients, and all patients should be screened routinely for this problem whenever type I APS has been diagnosed.

Type II APS

Type II APS is defined by Addison's disease plus an autoimmune thyroid disease and/or IDD. Type II APS usually presents later in adult life, and displays a female predominance which is often multigenerational.[94]

Type II APS is associated with other autoimmune diseases, such as late onset pernicious anemia, vitiligo, celiac disease, and myasthenia gravis (Table 3). Although a few individuals with type II APS have been reported to have gonadal failure, which is associated with high levels of circulating pituitary gonadotrophins, its occurrence is at least considerably less frequent than in type I APS. The occurence of chronic active hepatitis in type II APS is rare in contrast to its high frequency in type I APS.

Table 3. The frequency of autoimmune disorders in APS I and APS II.

Disease	APS I	APS II
Addison's Disease	83%	100%
Hypoparathyroidism	85%	
Mucocutaneous Candidiasis	73%	
Thyroid Disease	11%	69%
Insulin Dependent Disease	4%	52%
Chronic Active Hepatitis	13%	
Malabsorption	22%	
Alopecia	32%	0.5%
Pernicious Anemia	13%	0.5%
Gonadal Failure	17%	3.6%
Vitiligo	8%	4.5%

Most of the component diseases of APS II are strongly and primarily associated with DRB1*03/DQB1*0201 and secondarily with HLA-B8 through linkage disequilibrium.[94,95] These diseases are Addison's disease, IDD and Graves' disease. Only pancreatic beta cell autoimmunity and IDD are associated with DRB1*04/DQB1*0302.

Receptors and Enzymes as Autoimmune Targets

Receptors and enzymes have been identified as the targeted autoantigens in a number of autoimmune endocrinopathies.

There are a number of receptors that are autoantigens targeted by an immune response in organ-specific autoimmune diseases.[102] These include the thyroid stimulating hormone (TSH) receptors in autoimmune thyroid disease,[85] acetylcholine (ACh) receptors of skeletal muscle in myasthenia gravis,[81-83] gastrin receptors in pernicious anemia,[103] corticotropin receptors in Addison's disease,[104] and insulin receptors in IDD.[105]

The mechanisms for the involvement of receptors in autoimmune responses are probably complex. The first possibility is that autoantibodies against cell surface receptors may lead to functional abnormalities of the cells expressing them, resulting in receptor-mediated stimulation or

inhibition of the targeted cells. One example of stimulation by an agonist autoantibody mimicking a physiologic molecule is Graves' disease which is caused by the binding of autoantibody to TSH receptors such that they are stimulated but in a more prolonged manner than for TSH itself (long acting thyroid stimulator or LATS).[85,86] One example of inhibition by antagonist autoantibody is myasthenia gravis which is caused by the binding of antibody to ACh receptors.[81-84] The second possibility is that the autoantibodies cross-link the receptors and increase the rate of their degradation which ultimately leads to their depletion. The third possibility is that the autoantibody can bind to the receptor, fix complement, and thereby induce damage to the cells expressing the receptor. The remaining feature is that a cellular immune response often occurs as accompaning the autoantibody response, and lead to cell mediated lysis and target cell destruction.

A number of autoantigens have been found to be intracellular enzymes, e.g., 17α-hydroxylase[106] and 21-hydroxylase[107,108] in Addison's disease, thyroid peroxidase in autoimmune thyroiditis, H,K-ATPase in autoimmune gastritis,[109] tyrosinase in autoimmune vitiligo,[110] and glutamate decarboxylase in IDD.[111]

The mechanisms for involvement of intracellular enzymes in autoimmune responses are unknown. It is also interesting to

note that some of these autoantibodies recognize the catalytic site of the target enzyme. 17α-hydroxylase and 21-hydroxylase are such examples. It is unclear how antibodies have access to intracellular enzymes *in vivo* or how adrenal cell surface autoantigens identifed by immunofluorescence are related to those in the cytoplasm. Peripheral blood T-cell response to thyroid peroxidase and glutamic acid decarboxylase peptides have been described in Hashimoto disease and IDD, respectively. These findings raise the alternative possibility that production of autoantibodies against enzymes is a secondary phenomenon, the initiating event being T-cell mediated cytotoxicity directed against endogenous, enzyme-derived peptides co-expressed with MHC molecules on the target cells.

Specific Aims of This Research

The goal of this research is to characterize and identify the autoantigens in APS by employing immunoblotting, immunoprecipitation and eukaryotic expression systems. My study was designed with the following specific aims:

1. To amplify a potential candidate autoantigen (the tyrosinase) cDNA in autoimmune vitiligo by PCR for the purpose of expression in E.coli, and test the expressed product for autoantibody reactivity.

2. To confirm the autoimmune nature of acquired hypoparathyrodism and to identify the autoantigens involved.

3. To investigate the possibility of ZP3 as a potential autoantigen in human gonadal failure.

4. To characterize the nature of the autoantigens involved in alopecia.

CHAPTER 2
VITILIGO

Introduction

Human vitiligo is a common skin disorder characterized by areas of depigmentation due to loss of melanin-forming cells or melanocytes.[112] Whereas the etiology of vitiligo is generally not known, it is often associated with one or more autoimmune endocrinopathies such as IDD, Addison's disease, Graves' disease, and Hashimoto thyroiditis.[94] In addition, anti-melanocyte antibodies which can lyse cultured human melancotyes by both complement activation and antibody-dependent cellular cytoxicity, in patients with vitiligo, as well as in chickens affected by the disease have been reported.[113-115]

Using cultured melanoma cell lines and cultured human melanocytes, Naughton et al[116,117] demonstrated the occurrence of anti-melanocyte antibodies in patients with vitiligo by immunofluorescent staining and immunoprecipitation methods.

Vitiligo has also been studied extensively in the Smyth Chicken model. Melanocyte autoantibodies were detected in the sera of affected chicks several weeks prior to the expression of depigmentation, and the autoantibodies identified were

shown to bind to multiple melanocyte proteins of between 65 to 80 kDa.[118]

The key enzyme involved in melanin synthesis is tyrosinase with a calculated molecular weight of 62 kDa. However, the native form of tyrosinase has a molecular weight of approximately 70 kDa.[119] Tyrosinase has been identified to be an important T cell target in melanoma. Visseren et al[120] reported that tyrosinase specific autoreactive CTL precursors are present in the blood of healthy donors which can be activated *in vitro* by exposure to a synthetic nonapeptide (AA 369-377) of tyrosinase. It was suggested that autoreactive CTLs specific to tyrosinase can best be activated, when the antigens are presented in high amounts such as in the case of melanoma.

Since patients with vitiligo have the loss of integumentary melanocytes, the aim of the research is to explore the possibility that a key enzyme or enzymes involved in melanin synthesis could be important autoantigens in the disease pathogenesis. My role in this study was to amplify the tyrosinase cDNA by PCR for the purpose of expression in E.coli.

Materials and Methods

Amplification of Tyrosinase cDNA

The original tyrosinase freeze-dried clone was purchased from American Type Culture Collection (ATCC, Rockville, MD). The E.coli clone was grown up in Luria-Bertani (LB) culture medium containing ampicillin and the tyrosinase-containing plasmid was purified by a mini-prep kit from Promega (Alkaline lysis methods).

The purified plasmid was then used as template for PCR. Two primers were used to amplify the full-length human tyrosinase cDNA for expression as fusion proteins with glutathione S-transferase (GST). The primers were designed with Bam H1 and Eco R1 restriction sites on their 5' end and 3' ends respectively for directional subcloning. PCR was performed using a Perkin Elmer Cetus DNA thermal cycles in a total volume of 100μl. Each cycle consisted of 40 seconds denaturation at 94^{o}C, 1.5 minutes annealing at 55^{o}C and 3 minutes of chain extension at 72^{o}C. Following PCR, the overlaying oil was removed, a 10μl aliquot of PCR product was electrophoresed in 1% agarose gel in EtBr to visualize the PCR products.

DNA Sequencing and Expressing

The 1.6 kb PCR product was digested and ligated into pGEX-2T for expression in E. coli by colleagues in Dr. Maclaren's lab. Briefly, 1 μl of the ligation reaction was transformed into competent E. coli DH5α cells (GIBCO BRL, Gaithersburg, MD). The E. coli clones carrying the 1.6 kb tyrosinase insert were screened by plasmid mini-prep and identified by restriction digestion. Once the correct clone was identified, it was grown up again and the recombinant plasmid was purified using the QIAGEN Plasmid Maxi Kit (QIAGEN, Chatsworth, CA). The highly purified plasmid was sent to LAX Sequencing Technologies Inc and the insert was sequenced by Sanger's dideoxy-mediated chain termination method. The sequencing result confirmed that the 1.6 kb was indeed human tyrosinase. For expression, the E. coli clone containing tyrosinase insert was grown in LB(Luria-Bertani) medium containing ampicillin overnight at 37°C in a shaking incubator. The overnight culture was diluted 1/10 and incubation was continued for 2 hour. The expression of tyrosinase protein was induced by adding Isopropyl ß-D-thyogalactoside (IPTG).

Results

The amplified tyrosinase cDNA appears as a 1.6 kb band on 1% agarose gel electrophoresis as expected (Figure 1). The remainder of the amplified DNA was purified by Wizard PCR Preps DNA purification system from Promega (Madison, WI). An aliquot of the purified DNA was checked by agarose gel electrophoresis and the remaining purified DNA was digested and ligated into expression vector and the tyrosinase protein expressed as described above. The reactivity of tyrosinase with autoantibodies from the sera of patients with vitiligo was tested by immunoblotting by colleagues from Dr. Maclaren's lab. Among the 26 vitiligo sera tested, 61% of them were positive for tyrosinase autoantibodies.

Discussion

Although autoantibodies against melanocytes have been well documented in the literature, the pathogenic role of these autoantibodies remains unknown. Since tyrosinase is an intracellular enzyme, autoantibodies could not penetrate the cell and reach the enzyme intracellularly. Alternatively, the autoimmune attack to melanocytes could have been initiated by autoreactive T cells such as CTLs and autoantibodies to tyrosinase as well as other intracellular components of the

Figure 1. PCR amplification of human tyrosinase cDNA. EtBr stained agarose gel shows the PCR product amplified from human tyrosinase cDNA. The PCR product appears as 1.6 kb as indicated by DNA marker. M: DNA marker; T: tyrosinase.


kb
2.645
1.605
1.198
T
M

cell could be produced as a secondary event. Our present study confirmed the autoimmune nature of vitiligo, particularly in patients who also suffered from other autoimmune disorders.

CHAPTER 3
GONADAL FAILURE

Introduction

Gonadal autoantibodies are found in some patients with Addison's disease and hypogonadism. The autoantibodies react to steroid hormone producing cells of the adrenal cortex, placental syncytiotrophoblast, Leydig areas of testis, and the theca interna/granulosa layers of ovarian follicles.[121] These steroidal cell autoantibodies precede the onset of ovarian failure in patients with type I APS. The antigens targeted by steroidal cell autoantibodies have been suggested to be the combinations of the P450 side chain cleavage and 17α-hydroxylase enzymes.[122]

Anti-oocyte autoantibodies have also been detected in infertile women which appear to block the adherence and penetration of the sperm through the zona pellucida of the ova.[123] The zona pellucida is composed of three major glycoproteins: ZP1, ZP2 and ZP3.[124] ZP3, the primary sperm receptor at fertilization, has been identifed as an autoantigen in experimental murine autoimmune oophoritis,[125,126] a model of human premature ovarian failure. Therefore, the possibility that ZP3 could be an autoantigen in human gonadal

failure was investigated.

Materials and Methods

Patients

I examined sera from 13 patients with gonadal failure. All of them were female, some of them had antibodies to pituitary, some had antibodies to GAD. I also studied 8 normal disease-free controls. None of the normal controls had autoantibodies to pituitary, GAD, or thyroid antigens.

In vitro Translation and Immunoprecipitation

The cDNA clone of human ZP3 (under T7 promoter) was provided by Dr.Jurrien Dean (Laboratory of Cellular and Developmental Biology, NIDDK). The expression of ZP3 was examined by the positive control antibody (monkey anti-human ZP3) provided by Dr.Kenneth Tung, Dept. of Pathology, Univ. of Virginia.

The recombinant plasmid was propagated in E.coli and purified by the Magic™ Minipreps System (Promega). The ZP3 cDNA was transcribed and translated as described according to the manufacturer's instructions (Stratagene, La Jolla, CA). In brief, 1μg circular plasmid DNA was transcribed in a 100μl

reaction for 2 hours at 40°C, using SP6 RNA polymerase in the presence of RNAsin. The translation was done using a methionine-free rabbit reticulocyte lysate (Promega) in a 50 µl reaction using 20% of the synthesized RNA as substrate in the presence of 4 µl ^{35}S-methionine (10 mCi/ml) (Amersham, Arlington Heights, IL).

Once the translation reaction was complete, the translated products were examined by taking 5 µl aliquots mixed with 20 µl of SDS sample buffer. The samples were heated at 100°C for 3 minutes and subjected to a 10% SDS-PAGE. For autoradiography, the gels were dried and exposed to X-ray film (XAR-2 ready pack, Sigma, St Louis, MO) overnight at room temperature. Since ZP3 is a glycoprotein, canine pancreatic microsomes (Promega) were added to the translation reaction mixture in order to obtain the mature glycosylated receptor. For characterization of the autoantibody reactivities, the translated products (50,000 cpm) were incubated at 4°C overnight with 2 µl of sera diluted in PBS with a final volume of 100 µl. The immunocomplexes were washed three times with ice-cold PBS in the presence of either 1% or 0.5% Triton X-100 and incubated with protein A - Sepharose beads for another 45 minutes. After washing, 50 µl of the SDS gel loading buffer were added to the bead and boiled for 3 minutes. Autoradiography were performed as above.

Results

The full length ZP3 cDNA was translated as a 45-50 kDa double band. The 50 kDa band represents the glycosylated form of the protein since it appeared only in the presence of microsome membranes, particularly in the presence of 2 μl membranes. Both the glycosylated and non-glycosylated form of ZP3 were recognized by the monkey anti-ZP3 antibody (Figure 2). None of the patient's sera or normal controls reacted to ZP3 (Table 4).

These preliminary results indicated that autoantibodies against human ZP3 were not detected by this technique. Since some autoantibodies may have low affinity to their antigens and may dissociate under high concentrations of detergent, the immunoprecipitation was also performed using 0.5% Triton X-100. However, as shown in Figure 2, similar results were obtained as in Figure 3.

Discussion

Experimental autoimmune oophoritis can be induced by immunizing mice with peptide derived from ZP3.[125] Adoptive transfer experiment has shown that $CD4^+$ T cells specific for ZP3 peptide were able to induce the disease. I hope that

Figure 2. Immunoprecipitation of human ZP3.
The *in vitro* translated human ZP3 was immunoprecipitated by a monkey anti-ZP3 antibody (lane 1). The 45 and 50 kDa bands represent the non-glycosylated band and glycosylated form of ZP3 respectively. The serum from normal monkey (lane 2), patients with gonadal failure (lane 3-4) and healthy control (lane 5) did not react with ZP3.


kDa
50
45
5 4 3 2 1

Table 4. Autoantibody reactivity to ZP3 (Immunoblot).

Subject	Antibody to ZP3
Patients with APS I	0/4
Patients with APS II	0/4
Isolated gonadal failure	0/3
Normal control	0/8
Monkey anti-ZP3 serum	+
Normal monkey serum	-

Figure 3. Immunoprecipitation of human ZP3 with reduced amount of detergent.
The *in vitro* translated human ZP3 was immunoprecipitated by a monkey anti-ZP3 antibody (lane 1). The 45 and 50 kDa bands represent non-glycosylated band and glycosylated form of ZP3 respectively. Reduced amount of detergent (0.5%) was used in the washing step. The serum from normal monkey (lane 2), patients with gonadal failure (lane 3-4) and healthy control (lane 5) did not react with ZP3.


kDa
-50
-45
5 4 3 2 1

autoantibodies against ZP3 might also be present in the circulation of human patients with hypogonadism. However, ZP3-specific autoantibodies have not been demonstrated so far. Autoantibodies to zona pellucida were developed by immunizing the (C57BL/6 x A/J)F1(B6AF1) mice with a ZP3-derived peptide which is a known T cell epitope,[127] but does not contain a B cell epitope. Thus a pure T cell reactive peptide has elicited autoantibodies through immunizations against a region of ZP3 outside the T cell peptide. The authors termed the autoantibodies produced in this circumstance "amplified autoantibodies". They concluded that the B cells responded to endogenous ovarian antigen after activation of ZP3-specific T helper cells, and that the induced autoantibodies do not have to mirror the immunogen that initiates the autoimmune process. Immunofluorescence studies have confirmed that the autoantibodies reacted to zona pellucida. However, the authors did not show that the autoantibodies were directed specifically against ZP3 as the zona pellucida contains numerous other proteins. Other approaches such as immunoprecipitation or immunoblot using either biochemically purified or recombinant ZP3 should provide direct answers concerning the specificity of these autoantibodies.

Since autoantibodies bind to zona pellucida as confirmed by immunofluorescence, it is possible that ZP1 and/or ZP2 may also be involved in the autoimmune process. However, only ZP2

and ZP3 were transcribed in growing oocytes and neither of them has been detected in resting oocytes. Since ZP3 is the primary sperm receptor and ZP2 is secondary sperm receptor, Dr. Tung's group has mainly focused on ZP3 in animal studies.[125] Although there is no overall similarity in the amino acid sequences of ZP2 and ZP3, they share two common structural motifs at the C-terminal regions. Therefore, the possibility that immunization of ZP2 would also induce oophoritis in mice should be tested.

In the present study, I have attempted to demonstrate the ZP3-specific autoantibodies using *in vitro* translated ZP3 as antigen by immunoprecipitation. However, no autoantibodies were detected in any of the sera tested. The translated ZP3 appeared as a 45-50 kDa band as expected and the translated product was recognized by the monkey anti-ZP3 antibody. Therefore, the failure of detecting autoantibodies was likely not due to technical problems. One possible explanation could be that if the autoantibodies do exist, they may be present at very low titers, below the level of detection by this technique. Other approaches such as ELISA are usually useful in the detection of autoantibodies as initial screenings once an antigen has been very well characterized, purified and solubilized. However, ELISA is not a good assay for the initial investigation of candidate autoantigens unless the antigen is readily available as purified and soluble form. In

the case of ZP3, the protein was translated in the rabbit reticulocyte system and it is only suitable for immunoprecipitation because the translated ZP3 is radio-labelled but in a mixture of many other proteins. Another possibility is that the autoantibodies have very low affinity such that they may dissociate quickly upon binding to the antigen.

CHAPTER 4
ACQUIRED HYPOPARATHYROIDISM

Introduction

The Anatomy and Physiology of Parathyroid Gland

Calcium ions are essential for a wide variety of biologic functions. This include vital extracellular processes, such as blood clotting, intercellular adhesion, and skeletal integrity. This also include intracellular processes, such as the regulation of hormonal secretion, cell division, and cell motility.[128]

The homeostasis of calcium and phosphate concentrations in the extracellular fluid is maintained by a finely integrated regulation of their absorption from the intestine, reabsorption from the glomerular filtrate and mobilization from the skeleton. The parathyroid glands are the regulatory organs that mediate fine control of ionized calcium levels through a direct effect on skeletal in bone. At the same time, they activate vitamin D in the diet or following its formation in the skin after ultra violet light exposure, such that intestinal absorption of calcium/phosphate is indirectly promoted.[128]

Most people have four parathyroid glands situated close to the posterior surface of the lateral lobes of the thyroid gland. The combined weight of the four glands is approximately 120 mg. The parathyroid glands of children weigh approximately half this amount. The normal epithelial cells comprising the gland are of two types: the chief cells and the oxyphil cells. The chief cells are considered to be actively engaged in synthesizing hormone and to be the main source of parathyroid hormone (PTH). The oxyphil cells are derived from chief cells.[128]

PTH is a single chain polypeptide of 84 amino acids, produced by two enzymatic cleavages at the amino terminus from its preprohormone (115 amino acids). The direct function of PTH is to maintain the homeostasis of circulating ionized calcium and phosphate concentrations in the extracellular fluid within a very tight range. The synthesis and secretion of PTH are regulated by the extracellular calcium ion concentration which the parathyroid chief cells are very sensitive to.

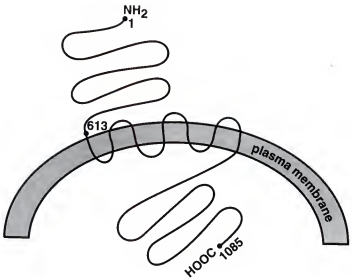
The Structure and Function of Ca-SR

The parathyroid chief cells are equipped with a Ca^{2+} sensing mechanism which exhibit an unusual inverse relationship between the Ca^{2+} levels and PTH release after

stimulation of its calcium sensing receptor (Ca-SR). The regulation of PTH secretion requires that parathyroid cells sense the free calcium ion levels in extracellular fluid. The recently cloned calcium sensing receptor responds to increased levels of extracellular calcium by triggering a phospholipase-C (PLC) dependent pathway which in turn induces the parathyroid cell to decrease its constituent PTH secretion.[129]

The Ca-SR was cloned by screening a cDNA expression library in Xenopus laevis oocytes.[130] The 5.275 kb cDNA has a 3.255 kb open reading frame encoding a protein of 1,085 amino acids with apparent molecular weight of 120-140 kDa. It contains a large extracellular domain of 613 amino acids at the amino terminus, a central core of 250 amino acids containing seven potential membrane-spanning helices characteristic of the G-protein coupled receptor superfamily and a intracellular domain of 222 amino acids at the carboxyl domain (Figure 4). The external domain probably serves as the actual ionized calcium detector. Sequence analysis shows that the Ca-SR contains 9 potential N-linked glycosylation sites. The Ca-SR gene for human has been mapped to chromosome 3q2. Mutations of the Ca-SR gene have been found to be responsible for familial benign hypocalciuric hypercalcemia (FBHH) and neonatal severe hyperparathyroidism.[131]

Figure 4. Diagram of the strategy for *in vitro* translation of Ca-SR.
The full length Ca-SR cDNA encodes 1085 amino acid. The extracellular domain (1-613) and intracellular domain (580-1085) were translated separately by a rabbit reticulocyte system.



PTH - secreting cell

The Detection of Autoantibodies in AH in the Past

An autoimmune etiology for AH has been suggested because of its association with other autoimmune diseases,[98] and by reports of autoantibodies directed against the parathyroid tissues in affected individuals. Autoantibodies to the parathyroid glands were first reported by Blizzard et al.[132] In that study, 38% of 74 patients with autoimmune hypoparathyroidism were found positive compared with only 6% of 245 healthy control subjects. The results from subsequent studies were controversial, since the antibodies often appeared to be directed against mitochondrial antigens which happen to be increased in parathyroid cells. Autoantibodies from the sera of patients with sporadic adult onset hypoparathyroidism however have been reported to bind to the cell surfaces of human parathyroid cells, resulting in an inhibition of PTH secretion.[133] In addition, autoantibodies in the sera of patients with AH have been reported to be cytotoxic for cultured bovine parathyroid cells, by an antibody mediated cytotoxicity dependent on complement fixation and activation.[134,135]

Whereas the above findings do constitute evidences for the presence of autoantibodies against parathyroid glands in AH, the target autoantigens have not been previously identified.

Materials and Methods

Patients

I examined sera from 25 patients with AH. Of these, 17 patients had APS I (all of them had AH, 14 had mucocutaneous candidiasis, 10 had Addison's disease, and many had associated vitiligo, alopecia, chronic active hepatitis and/or primary hypogonadism). Eight patients had adult-onset hypoparathyroidism associated with autoimmune hypothyroidism, confirmed by the presence of thyroid microsomal antibody and/or thyroglobulin antibody (Table 5). I also studied sera from 10 patients with Addison's disease, 10 with Graves' disease, 12 with Hashimoto's thyroiditis, 10 with insulin dependent diabetes (IDD) and 8 with vitiligo (none of whom had AH), as well as 22 normal disease-free controls. None of the normal controls had any endocrine-associated serum autoantibodies, such as thyroid microsomal, thyroglobulin autoantibodies, or islet cell autoantibodies.

Antigen Preparation

The human parathyroid glands were placed on ice in PBS with a mixture of protease inhibitors (1,10-phenanthroline, aprotinin, EDTA and benzamidine). The tissues were homogenized

Table 5. Characteristics of AH patients

Subject	Number	Gender	Age of Onset (range)
AH in APSI	17	10F & 7M	1 yr - 12 yr
AH in Adult	8	8F & 0M	31 yr - 53yr

AH, Acquired hypoparathyroidism; APS I, Type I autoimmune polyglandular syndrome; M, Male; F, Female.

with a glass tissue grinder and centrifuged at 15,000xg to remove cell debris, nuclei and mitochondrial proteins. The supernatant was centrifuged again at 100,000xg and the resulting supernatant (cytosolic) and pellet (membrane) fractions were used as antigen sources in the immunoblot and absorption studies.

Plasma membrane preparations from HEK-293 cells expressing the Ca-SR, together with membrane preparations from wild type HEK-293 cells were kindly provided by Dr.Forrest Fuller (NPS Pharmaceutical) and used as antigen sources in the immunoblot and absorption studies described below.

The cDNA clone of human parathyroid secretory protein (Chromogranin-A) was kindly provided by Dr. Helman-LJ (Molecular Genetics Section, National Cancer Institute). Monoclonal antibody to human chromogranin-A (LK2H10) was obtained from Boehringer Mannheim (Indianapolis, IN), rabbit anti-human chromogranin-A (A430) was from DAKO Corporation (Carpinteria, CA) and monoclonal antibody to human chromogranin-A (PHE5) was from Enzo Diagnostics (Farmingdale, NY). The negative control antibody anti-luciferase was from Promega (Madison, WI).

Immunoblotting

The parathyroid gland extract and the HEK-293 cell

membrane fractions were solubilized in SDS gel loading buffer containing DTT and heated for 3 minutes at 100°C before loading. After separation by a 8% SDS-PAGE, the proteins were transferred onto Immobilon-P membranes (Millipore, Bedford, MA). The strips of the membrane were cut and incubated with 1% BSA in Tris-buffered saline and 0.05% Tween-20 (TBST) to block free potential binding sites. Test sera at 1/100 dilutions as well as purified IgG of a rabbit anti-Ca-SR antisera and IgG of pre-immune rabbit sera were incubated with the antigen-containing strips. The strips were then incubated with an anti-human or anti-rabbit polyvalent immunoglobulin alkaline phosphatase conjugate, and developed with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT) (Promega, Madison, WI).

In vitro Translation and Immunoprecipitation

The human Ca-SR cDNA was obtained from Dr. Edward Brown (Brigham and Women's Hospital) and its extracellular and intracellular domains were amplified by PCR. The PCR products were positioned downstream of the SP6 promoter on the pcDNA3 construct. The recombinant plasmid was propagated in E.coli and purified by the Magic™ Minipreps System (Promega). The Ca-SR cDNA was transcribed and translated as described according to the manufacturer's instructions (Stratagene, La Jolla, CA).

In brief, 1μg circular plasmid DNA was transcribed in a 100μl reaction for 2 hours at 40^{0}C, using SP6 RNA polymerase in the presence of RNAsin. The translation was done using a methionine-free rabbit reticulocyte lysate (Promega) in a 50 μl reaction using 20% of the synthesized RNA as a substrate in the presence of 4 μl ^{35}S-methionine (10 mCi/ml) (Amersham, Arlington Heights, IL).

Once the translation reaction was complete, the translated products were examined by taking 5 μl aliquots mixed with 20 μl of SDS sample buffer. The samples were heated at 100^{0}C for 3 minutes and subjected to 10% SDS-PAGE. For autoradiography, the gels were dried and exposed to X-ray film (XAR-2 ready pack, Sigma, St Louis, MO) overnight at room temperature. Since Ca-SR is a glycoprotein, canine pancreatic microsomes (Promega) were added to the translation reaction mixture in order to obtain the mature glycosylated receptor.

For characterization of the autoantibody reactivities, the translated products (50,000 cpm) were incubated at 4^{0}C overnight with 2 μl of sera diluted in PBS with a final volume of 100 μl. The immunocomplexes were washed three times with ice-cold PBS and incubated with protein A - Sepharose beads for another 45 minutes. After washing, 50 μl of the SDS gel loading buffer were added to the beads and boiled for 3 minutes. Autoradiography were performed as above.

Absorption of Autoantibodies with Recombinant Ca-SR

The patient sera (2 μl) were incubated for 2 hours at room temperature with 1 mg recombinant Ca-SR expressed by HEK-293 cell diluted in 50 μl PBS. This mixture was then centrifuged (13,000g, 15 min), and the supernatant was again subjected to immunoprecipitation with the *in vitro* translated extracellular domain of the Ca-SR as an antigen source, to learn whether the positive band (see later) had been removed.

Results

The Identification of Autoantibodies in AH

Autoantibodies against specific proteins were detected in both cytosolic and membrane fractions of the parathyroid extracts.

(1) Autoantibodies to the cytosolic fraction of parathyroid gland.

Seventeen AH patient's sera were tested for their reactivity to the cytosolic fraction using immunoblot (Figure 5). Twelve (11 had APS I, 1 had adult onset AH) patient sera reacted to a protein of 70 kDa, and 16 (15 had APS I, 1 had adult onset AH) to one of 80 kDa.

Sera from 50 patients with other autoimmune diseases as

Figure 5. Immunoblot analysis using the cytosolic fraction of human parathyroid gland extract.
The cytosolic fraction of the parathyroid gland extract was solubilized and separated by a 10% SDS-PAGE. After separation, the proteins were transferred onto Immobilon-P membranes. Immobilon - P strips containing the parathyroid extract were incubated with AH sera (lanes 1-10) and control sera (lanes 11-12). The reactivities of the autoantibodies were visualized by an alkaline phosphatase mediated BCIP/NBT system. The autoantibodies from AH sera reacted to specific antigens with molecular weight of 70 kDa (lanes 1,2,3,4,5,6,8) and 80 kDa (lanes 1-9). Two patients's sera also reacted to a 60 kDa antigen (lanes 4 and 5). Only one of the AH patients (lane 10) was negative for the specific antigens.


200 kDa
80 kDa
70 kDa
1 2 3 4 5 6 7 8 9 10 11 12 M

well as 13 normal controls were also tested, and none of them reacted with any of the above specific parathyroid proteins (Fig.6).

Since human parathyroid secretory protein (PSP) also known as chromagranin A has a molecular weight which is similar to the 70 kDa antigen, I investigated the possibility that the PSP is a potential antigen. The PSP cDNA was translated *in vitro* under the control of the SP6 promotor. The translated protein has a molecular weight of 68 kDa and it appears as a non-glycosylated protein (Figure 7).

The translated PSP was verified by positive control antibodies raised against this protein as shown in Figure 8. Monoclonal antibody PHE5 only shows a faint band for PSP, whereas monoclonal antibody LK2H10 and rabbit antibody A430 show clear staining of the 68 kDa PSP protein. A430 also reacted to the 60 kDa protein which might be a truncated form of the PSP. The negative control antibody (anti-luciferase) did not react to PSP. Next, the reactivity of the sera from patients with AH to PSP was tested. As shown in Figure 9. None of patients sera reacted to PSP.

(2) Autoantibodies to the membrane fraction of parathyroid gland.

Autoantibodies were detected against the membrane fraction of parathyroid gland in 5 of 25 (20%) of the AH sera by immunoblot. Two patients had APS I and 3 had adult onset

Figure 6. Immunoblot analysis of the cytosolic fraction of human parathyroid gland extract using normal human sera. The cytosolic fraction of the parathyroid gland extract was solubilized and separated by a 10% SDS-PAGE. After separation, the proteins were transferred onto Immobilon-P membranes. Immobilon - P strips containing the parathyroid extract were incubated with normal human sera (lanes 1-13). The reactivities of the autoantibodies were visualized by an alkaline phosphatase mediated BCIP/NBT system. The normal human sera reacted to a non-specific band with molecular weight of 90 kDa.

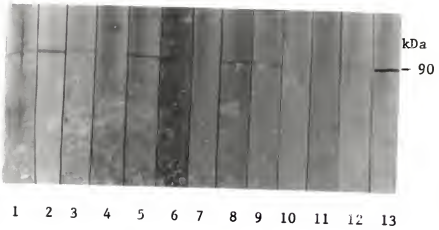

kDa
90
1 2 3 4 5 6 7 8 9 10 11 12 13

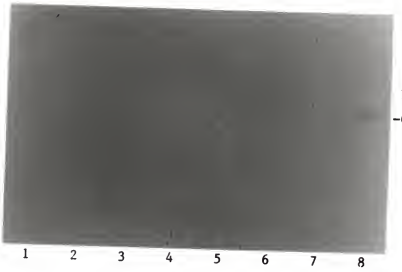
Figure 7. *In vitro* translation of human parathyroid secretory protein (PSP).
The *in vitro* translated products were separated by a 12% SDS-PAGE and then visualized by autoradiography. The human PSP cDNA was translated as a 68 kDa protein and a minor band of 60 kDa. Addition of microsomal membranes (lanes 2-3) did not cause additional bands indicating that the PSP is not a glycoprotein.


kDa
-68
1
2
3

Figure 8. Immunoprecipitation of human parathyroid secretory protein (PSP) by control sera.
The *in vitro* translated PSP was incubated with monoclonal antibodies against human PSP (lane 1: PHE5, lane 2: LK2H10), rabbit anti-human PSP (lane 3: A430) and negative control antibody (lane 4: rabbit anti-luciferase).


kDa
-68
1
2
3
4

Figure 9. Immunoprecipitation of human parathyroid secretory protein (PSP) by sera from patients with hypoparathyroidism. The *in vitro* translated human PSP was immunoprecipitated by patient's sera (lanes 1-6), normal human sera (lane 7) and positive control sera LK2H10 (lane 8). Only the positive control sera reacted to PSP.


kDa
-68
1
2
3
4
5
6
7
8

AH. The autoantibodies reacted with a doublet 120-140 kDa protein in the parathyroid gland extract (Figure 10). Sera from 50 patients with other autoimmune diseases as well as 22 normal controls were also tested, and none of them was positive.

The Characterization of Ca-SR in AH

Since the parathyroid 120-140 KDa antigen has the same molecular weight as the Ca-SR dependent upon its degree of glycosylation, I tested the possibility that the receptor itself was the autoantigen by three different experimental approaches.

In the first approach, the AH sera were tested by immunoblot using a membrane fraction of HEK-293 cells transfected with Ca-SR cDNA. The patient sera reacted to a 120-140 kDa protein (Figure 11, lane 2), which closely matched that recognized by the anti-Ca-SR IgG raised in rabbit (Figure 11, lane 3). The patient sera at 1:100 dilution did not react with other bands. Eight of 25 AH patient sera (32%, 3 APS I and 5 adult onset AH) including the above-mentioned 5 positive sera reacted to the Ca-SR from this source, but none of the control sera did so. In addition, the 8 positive AH patient sera did not react to non-transfected or wild type HEK-293 cells which did not express Ca-SR proteins (Table 6).

Figure 10. Immunoblot analysis using the membrane fraction of human parathyroid gland extract.
The membrane fraction of the parathyroid gland extract was solubilized and separated by a 8% SDS-PAGE. After separation, the proteins were transferred onto Immobilon-P membranes. Immobilon - P strips containing the parathyroid extract were incubated with normal sera (lane 1) and AH sera (lane 2). The reactivities of the autoantibodies were visualized by an alkaline phosphatase mediated BCIP/NBT system.

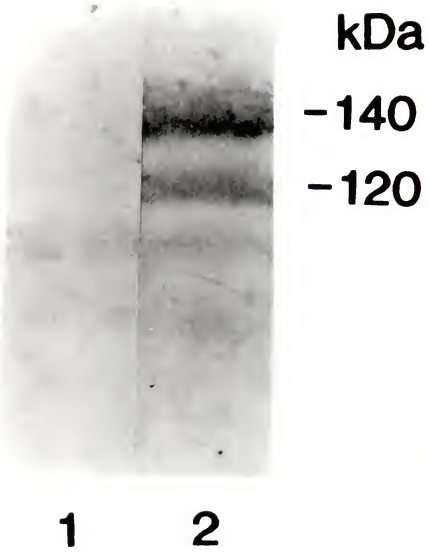

kDa
-140
-120
1
2

Figure 11. Immunoblot analysis using membranes of HEK-293 cells transfected with human Ca-SR cDNA.
The HEK-293 cell membranes were solubilized and separated by 8% SDS-PAGE, then transferred onto Immobilon-P membranes. Immobilon - P strips containing the antigen were incubated with normal sera (lane 1), AH sera (lane 2), rabbit anti-Ca-SR IgG (lane 3) and pre-immune rabbit IgG (lane 4). The reactivities of the antibodies were visualized by an alkaline phosphatase mediated BCIP/NBT system.

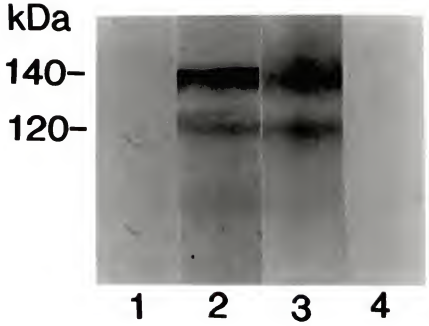

kDa
140–
120–
1
2
3
4

Table 6. Autoantibody reactivity to recombinant Ca^{2+}-Sensing receptor (Immunoblot).

Ag source	AH patients	Normal	Rabbit anti-Ca-SR IgG
Transfected HEK-293 cell	8/25 (32%)	0/22	+
Wild type HEK-293 cell	0/25	0/15	-

AH: Acquired hypoparathyroidism.

In the second approach, the Ca-SR was translated *in vitro* into two parts in order to identify the antigenic epitopes reactive to the putative autoantibody that we had discovered. Overlapping extracellular (residues 1-613) and intracellular (residues 580-1085) domains of the Ca-SR were expressed as shown in Figure 4. The extracellular domain was translated as shown in Figure 12. Two bands with the molecular weight of 46, and 60 kDa are seen in lane 1. Glycosylation occurs with the addition of canine pancreatic microsome membranes. As can be seen in lanes 2-5, this step induced one additional band to appear as 70 kDa, meanwhile, the intensity of the 60 kDa band decreased by 50% as expected for glycosylated proteins. Apparently, the 60 kDa is the non-glycosylated form and 70 kDa is the glycosylated form of extracellular Ca-SR. The 46 kDa band, however was unexpected. To determine whether it was a form of Ca-SR or an unrelated artifact of the translation system, I immunoprecipitated the translated extracellular domain by the rabbit anti-Ca-SR antibody. This rabbit antibody was raised against a peptide of the extracellular domain. As shown in Figure 13, the antibody recognized all three bands, suggesting that the 46 kDa band is also a portion of the Ca-SR, perhaps a degraded or truncated product.

The patient sera reacted to 60 and 70 kDa forms of the extracellular domain but not the 46 kDa band (Figure 14) indicating that the autoantibodies recognized different

Figure 12. *In vitro* translation of the extracellular domain of the Ca-SR.
The *in vitro* translated products were separated by a 10% SDS-PAGE and then visualized by autoradiography. The extracellular domain of the Ca-SR was translated as 46 and 60 kDa protein bands (lane 1). One additional band (70 kDa) appears when 1-4 μl of microsomal membranes were added to the reaction (lanes 2-5).

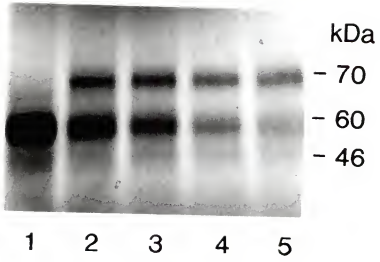

kDa
70
60
46
1
2
3
4
5

Figure 13. Immunoprecipitation of the extracellular domain by rabbit anti-Ca-SR.
The *in vitro* translated extracellular domain of the Ca-SR was incubated with a rabbit anti-Ca-SR IgG (lane 1) or with a pre-immune rabbit IgG (lane 2). Samples were precipitated by protein-A-Sepharose, separated by a 10% SDS-PAGE and then visualized by autoradiography.

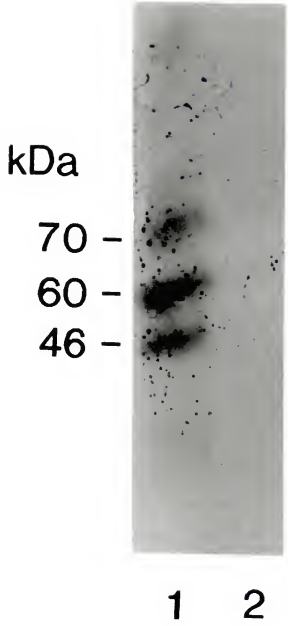
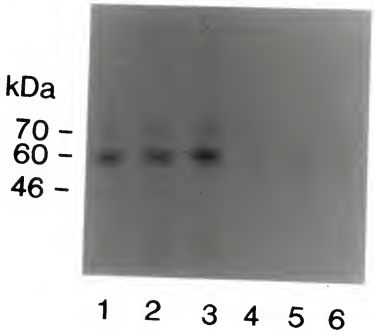

kDa
70 -
60 -
46 -
1
2

Figure 14. Immunoprecipitation of the extracellular domain by AH sera.
The *in vitro* translated extracellular domain of the Ca-SR was incubated with AH sera (lanes 1-3) or with normal control sera (lanes 4-6). Samples were precipitated by protein-A-Sepharose, separated by 10% SDS-PAGE and then visualized by autoradiography.


kDa
70 -
60 -
46 -
1 2 3 4 5 6

epitopes compared to the rabbit antibody. By using this technique, 14 of 25 (56%) AH sera were positive. Six had APS I and 8 had adult onset AH. Furthermore, glycosylation is not required in the formation of all autoantibody reactive antigenic epitopes since both the non-glycosylated (60 kDa) and glycosylated (70 kDa) proteins were recognized. None of the control sera reacted to the extracellular domain of the Ca-SR.

In the third approach, the positive AH sera were pre-incubated with the HEK-293 membranes containing the Ca-SR. The reactivity of the sera was completely removed after the pre-absorption. As shown in Figure 15, the AH sera reacted with a 60 and 70 kDa protein before absorption (lane 1) and the reactivity disappeared after the absorption (lane 2).

The cytosolic domain was translated as a 60 kDa protein and no glycosylation occurred after exposure to the microsomal membranes as expected. None of the patient sera reacted with the cytosolic or intracellular domain of the Ca-SR (Table 7).

In summary, 14 (56%) of AH patient sera reacted to the extracellular domain of the recombinantly expressed Ca-SR, whereas none of the 25 AH patient sera reacted to the intracellular domain of the molecule. The 14 antibody positive patients which responded to the extracellular domain of the Ca-SR included all 8 positive patients that had reacted to the transfected HEK- 293 cells. The autoantibody frequencies might

Figure 15. Absorption studies with HEK-293 cells.
The extracellular domain of the Ca-SR was immunoprecipitated by a positive AH sera before (lane 1) and after (lane 2) pre-absorption with HEK-293 cell membranes containing the Ca-SR. Samples were precipitated by protein-A-Sepharose, separated by a 10% SDS-PAGE and then visualized by autoradiography.

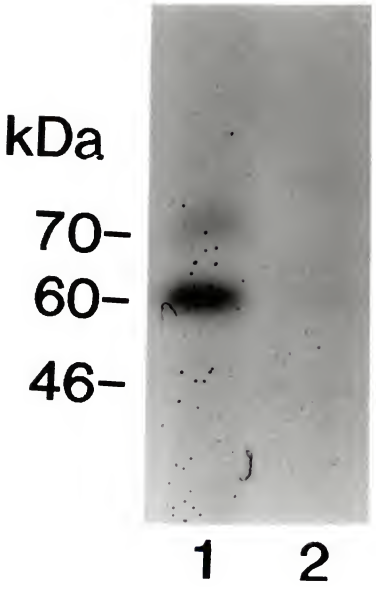

kDa
70–
60–
46–
1
2

Table 7. Autoantibody reactivity to *in vitro* translated domains of calcium sensing receptor (immunoprecipitation).

Subject	Antigen Source	
	Ca-SR Extracellular	Ca-SR Intracellular
AH	14/25 (56%)	0/25
Addison's disease	0 /10	0/10
Graves' disease	0 /10	0/10
Hashimoto thyroiditis	0 /12	0/12
IDD	0 /10	0/10
Vitiligo	0 /8	0/ 8
Normal control	0 /22	0/22

Ca-SR, calcium sensing receptor; IDD, insulin dependent diabetes; AH, acquired hypoparathyroidism.

have been higher if newly diagnosed patients had been exclusively studied. None of the 22 normal control sera reacted to either domain of the Ca-SR. Sera from 50 patients with other autoimmune diseases were also tested, and none of them reacted to either domain of the Ca-SR (Table 7).

Discussion

Our studies confirm the autoimmune nature of AH and demonstrate that autoantibodies in patients with AH target human parathyroid proteins of 70, 80 and 120-140 kDa. The autoantigens are disease specific since they were only recognized by the sera from patients with AH and not from those with other autoimmune diseases.

The autoantigens are not mitochondrial proteins, because the mitochondrial proteins were removed by centrifugation prior to immunoblotting in our study. Furthermore, we chose fresh human hypercellular parathyroid glands and normal dog parathyroid glands instead of parathyroid cell lines or tumors, so that we could avoid spurious identification of any antigens that are not normally present in the parathyroid glands.

Most of the Ca-SR autoantibody positive patients (including 5 AH in the context of APS I and all 8 adult-onset AH in association with thyroid disease) were females (Table

8). This finding of female predominance is consistent with results in other autoantibody mediated diseases targeted at membrane receptors. Four of our adult-onset AH patients developed their disease and had the Ca-SR autoantibodies detected after they had babies, another 2 adult-onset AH patients developed their disease after menopause, while one who presented with AH in the context of APS I began her disease at the onset of her menses. These findings suggest a possible influence of female hormones in the manifestation of the disease, as in many other autoimmune syndromes.

That autoantibodies to Ca-SR were absent from some AH in the context of APS I could be possibly explained by the complete loss of the autoantigen needed to drive their formation long before we could study them. Two of the Ca-SR autoantibody negative AH patients had developed their disease 32 years ago, while another 2 autoantibody negative AH patients had their diseases for more than 10 years at the time of this study. However, we were able to collect a serum sample immediately after the onset of AH from a 34 years old female who developed AH after an infection by influenza and she happened to be strongly positive for Ca-SR antibody. A general characteristic of all autoimmune diseases is that there are remissions and exacerbations of the underlying pathogenic processes involved over time. With IDD, islet cell autoantibodies (ICA) disappear following clinical onset of

Table 8. Characteristics of positive AH patients.

Subject	Number	Gender	Positive *in vitro* translation
AH in APSI	17	10F & 7M	5F & 1M
AH in Adult	8	8F & 0M	8F & 0M

AH, Acquired hypoparathyroidism; APS I, Type I autoimmune polyglandular syndrome; M, Male; F, Female.

disease when the pancreatic ß cells are destroyed, and the ICA reactive self antigens have disappeared. In some diabetic patients, even with the combination of different well defined antigens, autoantibodies are never detected. AH may have a similar course in respect to the Ca-SR autoantibody. Alternatively, different antigens may exist in different patients or it is possible that in some patients they simply do not appear at any time. Finally, the Western blot technique I used, although specific for antigen reactivity, is a relatively insensitive method for detecting autoantibodies. Other more sensitive assays, such as radioimmunoassay or ELISA, may be able to increase the autoantibody frequencies.[110] This possibility remains to be explored.

The Ca-SR appeared as a 120-140 kDa bands on immunoblot and the external domain of Ca-SR appeared as a 60-70 kDa bands on immunoprecipitation due to differential glycosylation of the receptor components. However, this differential glycosylation did not appear to affect the antigenic structure of the Ca-SR since both the 120-140 kDa and 60-70 kDa bands were well recognized by both the rabbit antibody and the AH patient sera. The transfected HEK-293 cells contain more Ca-SRs than the normal human parathyroid gland membrane preparations and this may explain why more of the AH patients were found to be positive when the HEK-293 membranes rather than human parathyroid gland membranes were used as antigen

sources in immunoblotting (Table 9). In addition, the *in vitro* translated external domain of Ca-SR had much less background than the transfected HEK-293 cells and this may explain why as many as 56% of the AH patients were found to be positive when *in vitro* translated external domain of Ca-SR was used as the antigen source in immunoprecipitation studies.

I have yet to identify the 70 and 80 kDa parathyroid protein antigens. Parathyroid secretory protein (PSP) has a molecular weight of 70 kDa and is co-stored and co-secreted with PTH in the parathyroid gland.[136] The protein has been shown to be present in the secretory granules of a variety of neuroendocrine and endocrine tissues with greatest abundance in parathyroid glands and adrenal medulla. I therefore tested the reactivities of the positive sera with PSP using the *in vitro* translation based immunoprecipitation technique, but found no positive reactivities.

Many membrane proteins and serum proteins contain carbohydrate chains called glycoproteins, which often contribute importantly to the folding and stability of the proteins as well as to their synthesis and positioning within a cell, especially as integral membrane proteins. Sugar residues in glycoproteins are commonly linked to two different classes of amino acid residues. The sugars are classified as O-linked if they are bonded to the hydroxyl oxygen of serine, threonine, and (in collagen) hydroxylysine; whereas they are

Table 9. Autoantibody reactivity to Ca-SR.

Subject	Antigen source		
	Gland	HEK-293	*In vitro* translation
AH in APS I	2/17	3/17	6/17
AH of adult onset	3/8	5/8	8/8
Ratio	20%	32%	56%

classified as N-linked if they are bonded to the amide nitrogen of asparagine. It is possible that the 70 and 80 kDa autoantigens represent the same molecule with differential glycosylation. To test this hypothesis, the cytosolic fractions of the parathyroid gland have been incubated with endoglycosidase H and O-glycosidase which cleave N- and O-linked oligosaccharide respectively. After incubation, the enzyme treated cytosolic fraction was used as antigen source for immunoblot. The 70 and 80 kDa antigen were found unchanged after this treatment indicating that they are not glycoprotein. This would be consistent with their cytosolic location.

There are still other approaches to identify the nature of the 70 and 80 kDa antigen. One of the approaches would be to purify and sequence the protein and design a DNA probe according to the partial amino acid sequence of the proteins and then use the probe to screen a cDNA library. The cytosolic fraction was separated by SDS-PAGE and stained with Commasie brilliant blue. The 70 and 80 kDa region were crowded by multiple minor bands. No dominant band in sufficient quantity could be isolated for sequencing purpose. This result indicated that the 70 and 80 kDa protein may not have a dominant function unique to the parathyroid cells.

The role of Ca-SR autoantibodies in the pathogenesis of AH is not known. The positive patient sera were sent to Dr.

Fuller's group to test this possibility on the HEK-293 cells transfected with Ca-SR. This established transfected cell line expresses Ca-SR on the plasma membrane and responds to extracellular calcium by increasing the intracellular Ca^{2+} level. Our positive sera did not show any effect on the intracellular Ca^{2+} level in this cell line. This result indicates that the autoantibody against Ca-SR may not have an functional effect, instead, it may fix complement to lyse the cell or just be an indicator of the autoimmune process. The pathogenic event might also involve cytotoxic lymphocytes rather than autoantibodies. The specificity of our results however argue best for a possible role of autoimmunity to the Ca-SR in AH.

Our detection of autoantibodies to the Ca-SR in AH could lead to the development of a diagnostic test for the disease, as well as possibly provide antigen mediated immunotherapies based upon the use of recombinant protein antigen as a therapeutic agent to restore immune tolerance in AH.

CHAPTER 5
ALOPECIA

Introduction

Alopecia areata is a common condition that results in the loss of hair on the scalp and elsewhere. Potentially reversible, it is characterized by either limited patchy hair loss (alopecia areata, patchy AA), loss of all scalp hair (alopecia totalis, AT),or loss of all body hair (alopecia universalis, AU).[137]

Alopecia occurs in males and females of all ages, but young persons are affected most often. The incidence of alopecia areata has been reported at about 17 per 100,000 per year, therefore approximately 1% of the population will have been affected by the age of 50.[138]

The etiology of alopecia areata is not clear. Occasional unexplained outbreaks of alopecia areata in closed communities have been reported and it has been postulated that viral infection might serve as a trigger mechanism for an imbalance in a T-cell subpopulation provoking onset of alopecia areata. It is known that stress can profoundly affect the immune system and many physicians feel that psychological factors may play a part in alopecia areata.

AA has been suggested to be an autoimmune disease because of its association with other autoimmune disorders,[139,140] an inflammatory infiltrate of activated T cells surrounding the hair follicles (HFs) in affected areas, and deposits of immunoglobulin and complement around HFs particularly at the edge of active lesions. Recent studies have shown that serum from patients with alopecia areata significantly inhibited the growth of normal dermal papilla cells, implicating a serum factor reacting to dermal papilla cells.[141]

Cortisone injection and the application of anthralin cream are being used for mild patchy alopecia areata. Topical immunotherapy is being used for extensive alopecia areata, or alopecia totalis/alopecia universalis. Chemicals such as dinitrochlorobenzene (DNCB) or diphencyprone (DPCP) are applied to the scalp to produce an allergic rash which resembles poison oak or ivy. Approximately 40% of patients treated by this way will regrow scalp hair. The mechanism for this treatment is not known. Recent studies from Happle et al[142] has indicated that the beneficial effect of DPCP may be mediated by cytokines locally released during the contact allergy. They perfomed a semiquantitative RT-PCR with RNA extracted from scalp biopsies that were obtained from patients with AA before and after successful treatment with DPCP. They found an increased mRNA levels for IL-2, IL-8, IL-10 and TNF-α. The overall expression level for IFN-gamma was reduced by

60% compared to untreated AA. These studies provided experimental evidence that cytokines may participate in the pathogenesis of AA and that T cells might trigger the hair loss by releasing IFN-gamma.

Autoantibodies against normal human hair bulbs have been recently demonstrated by Dr. Maclaren's group using indirect immunofluorecense technique. Following this finding, I started to investigate the potential alopecia autoantigens using immunoblot and immunoprecipitation techniques.

Materials and Methods

Antigen Preparation

A portion of the same crown head human skin as used in immunofluorescence was homogenized on ice in the presence of 1% Nonidet P-40 and protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 mM aprotinin, 10 mM leupeptin, 10 mM iodoacetamide). After homogenization, the homogenate was incubated on ice for 30 mins. The total homogenate was used directly for immunoblotting. In addition, the skin homogenates were centrifuged at 13,000 g for 30 min at 4°C and the supernatant was used for immunoprecipitation.

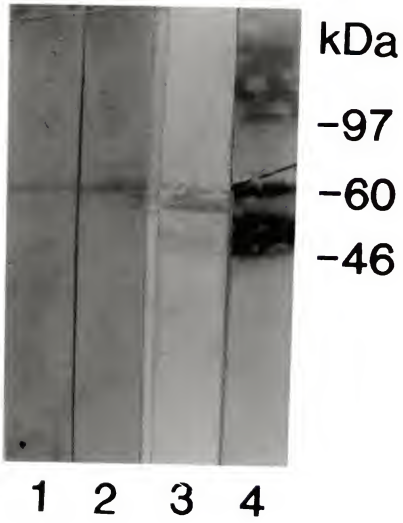
Immunoblotting

The skin homogenates were boiled in SDS-loading buffer and loaded to a 12% SDS-PAGE. The separated proteins were transferred onto Immunobilon-P membrane by a semi-dry electrotransfer unit. Immobilon-P strips were incubated with 1% BSA in Tris-buffered saline and 0.05% Tween-20 to block free binding sites. Test sera at 1/100 dilutions were incubated with the antigen-containing strips. The strips were then incubated with an anti-human polyvalent immunoglobulin alkaline phosphatase conjugate, and the indicator colour developed with 5-bromo-4-chloro-3-indolyl phophate (BCIP) and nitro blue tetrazolium (NBT).

Results

The two most positive patient sera, together with two normal controls were initially analyzed. The skin extract was quantitated by Bradford's method, and 200 μg of protein were loaded on the gel. As can be seen from Figure 16, both control (lanes 1-2) and patient sera (lanes 3-4) reacted to a protein of 60 kDa. The 60 kDa protein thus appeared to be a non-specific antigen for the disease. However, the patient sera also reacted specifically to additional proteins. Both patient sera (lanes 3-4) reacted to three identical proteins

Figure 16. Immunoblot analysis using human head skin homogenate.
Aliquot of the total human head skin homogenate was solubilized and separated by a 12% SDS-PAGE. After separation, the proteins were transferred onto Immobilon-P membranes. Immobilon - P strips containing the head skin extract were incubated with control sera (lanes 1-2) and alopecia sera (lanes 3-4). The reactivities of the autoantibodies were visualized by an alkaline phosphatase mediated BCIP/NBT system.


kDa
-97
-60
-46
1
2
3
4

with molecular weights between 46 to 55 kDa. These two positive sera also reacted to different proteins in addition to the common ones. One of them (lane 3) reacted to a protein of 57 kDa, the other serum (lane 4) reacted to several proteins of high molecular weight (above 100 kDa).

In addition to the above-mentioned two patient sera, I also tested 9 other AA sera by immunoblot. Four of the 9 sera were positive by this method. All of the four positive sera reacted to 46 - 55 kDa antigens (Table 10). Three of the 11 AA sera also reacted to the high molecular weight proteins (>100 kDa).

There is a good correlation between the immunoblot and immunofluoresence findings. Sera that stain all layers of the hair follicle on immunofluoresence reacted to both high and low molecular weight antigens. Those that stain only the outer layer of HF reacted only to the low molecular weight antigens (46 - 55 kDa). These results indicate that the antigens may be located in different regions of the HFs or different types of cells may be attacked in the autoimmune process.

Discussion

Autoantibodies directed to human hair follicles (HFs) have been demonstrated recently by Tobin and Bystryn et al.[143] Autoantibodies reacted to specific autoantigens derived from

Table 10. Autoantibody reactivity to human head skin homogenate.

Antigen	Patients with alopecia
46 - 55 kDa	6/11
>100 kDa	3/11
57 kDa	1/11

keratinocytes and melanocytes derived from HFs but not from the same type of cells from epidermis. In their immunoblot assay, the autoantibodies to HF keratinocytes reacted to antigens with molecular weight of 48-50, 52-54, 58-60, and 62-64 kDa. The same research group has also developed a mouse model for alopecia. The C3H/HeJ mice develop alopecia with age that quite closely resembles human alopecia. The mice autoantibodies reacted to antigens of approximately 46, 50, 55, 60 and/or 64 kDa. The autoantigens identified so far by this group matched our findings, at least in respect to the 46-55 and 57 kDa antigens. Most interestingly, autoantibodies to some of these antigens in the mice were detected in the littermates who had not yet developed hair loss. The authors concluded that the autoantibodies may be a cause rather than a result of hair loss. An autoantibody transfer experiment will be needed to establish the causal role of the autoantibodies.

According to the literatures, no proteins which have similar molecular weights to these autoantigens and are unique for melanocyte or keratinocyte have been found so far. Therefore, I am not able to speculate a candidate autoantigen at this moment.

The nature of these autoantigens will be identified by a different approach. My next step is to get partial amino acid sequence of the above-mentioned antigens. DNA probes will be

designed based on the knowledge of the amino acid sequence, and the probes will be used to screen a cDNA library generated from human skin mRNAs.

CHAPTER 6
GENERAL DISCUSSION

Since the initial demonstration of organ specific autoantibodies by Doniach and Roitt in 1957, autoimmune processes involved in endocrine diseases have been extensively investigated. With the rapid development of recombinant DNA technology, particularly during the last decade, many of the major autoantigens involved in autoimmune endocrine diseases have been identified as summarized in Table 1. Studies on the correlation between the autoantibodies and the disease processes have been fruitful in terms of prediction and prevention of some of the diseases. While much attention has been focused on the major endocrine diseases such IDD and autoimmune thyroid diseases, less information is available about type I APS. The topic of the autoimmune polyglandular syndromes has long been of interest to both endocrinologists and immunologists. Collectively, these diseases are very common. My thesis has focused on the identification of autoantigens involved in hypoparathyroidism and associated diseases such as vitiligo, gonadal failure and alopecia areata, all of which are component diseases of type I APS.

A scientific issue that needed to be resolved was whether multiple glandular autoimmunities affected individual patients

and their families because they made autoimmune responses to a single antigen commonly present in multiple tissues. This possibility appears unlikely, as exemplified by our findings as well as those of others. The autoantigens all seem different for the component diseases as summarized in Table 1. One exception is 17α-hydroxylase and P450scc which are found in steroid cells of adrenal cortex, testes and ovary. Organ specific autoantigens may also share common motifs with each other or with invading microorganisms. In fact, 21-hydroxylase and 17α-hydroxylase share a common motif around the steroid binding site, however, autoantibodies from patients with Addison's disease reacted only with the 21-hydroxylase. Autoimmune reactivity to common motifs of unrelated proteins has been demonstrated at T cell level. A nonamer peptide from murine nicotinic acetylcholine receptor which shares four amino acid residues with a nonamer peptide of murine ZP3 was able to induce murine autoimmune oophoritis.[126]

Knowledge of the nature of the autoantigens involved in APS now allows us to speculate about the dominant pathogenic events involved. In the case of type I APS, a Th2 like antibody mediated process may be operating as documented by my recent identification of the external domain of the calcium-sensing receptor as a major autoantigen in hypoparathyroidism. In addition, researchers from two different groups have shown that IgG from patients with type I APS and Addison's disease

inhibited ACTH-stimulated cortisol secretion by guinea pig adrenal cells. Although direct immunological evidence (e.g., immunoprecipitation) is lacking, it is logical to think that the targeted autoantigen in this case may be the ACTH receptor. Anti-FSH receptor antibodies have also been reported in the literature. On the other hand, type II APS appears to be mediated by autoreactive $CD8^+$ cytotoxic T cells (a Th1 like cellular immune response). The component diseases with evidence for this include IDD, Hashimoto's thyroiditis and vitiligo. Graves' disease has long been considered to be mediated by TSHR-Ab, however, the role of autoantibodies in Graves' disease has been questioned since they show only poor correlations with the disease process and it is now believed that autoreactive T cells contribute to the pathogenesis of the disease. Addison's disease may be mediated by a Th1 like response in the context of type II APS, whereas a Th2 like response may be responsive if the disease occurs in the context of type I APS.

If anti-Ca-SR autoantibody is pathogenic, its mechanism of disease initiation is unknown. Autoantibody to acetylcholine receptor (AChR) is a good example of pathogenic autoantibody. This autoantibody is present in most patient with myasthenia gravis (MG). Although a significant number (>10%) of MG patients including some with severe generalized weakness have no detectable autoantibodies to AChR, the

pathogenic role of this antibody have been well established. Elimination of the autoantibody by immunosuppression therapy resulted in marked improvement in the majority of MG patients. It is known that the AChR is lost at the motor end-plates due to the autoantibodies. The exact way that the autoantibodies exert their effect is not known. The antibody may cross-link AchR and increase the rate of receptor degradation or antibody may fix complement.

Although autoantibodies may not be pathogenic in type II APS, the detection of the autoantibodies may provide valuable marker tools for clinical diagnosis and possible prevention of the diseases. The identification of the reactive autoantigens has made this possible. In IDD, beta cells of the pancreas are destroyed as the result of an autoreactive T cell attack. Autoantibodies to islet cell antigen may be produced as a secondary event which may occur several years before the onset of diabetes. The detection of these autoantibodies has proven to be very valuable in identifying prediabetic patients and therefore permitting the design of immunological intervention at an early stage.

In summary, hypoparathyroidism is a major component disease of autoimmune polyglandular syndromes. The autoimmune nature of hypoparathyroidism has been confirmed by the detection of specific autoantibodies and the identification of the calcium sensing receptor as an autoantigen is the first

step toward the understanding of the autoimmune process of the disease. Alopecia is a non-endocrine disease associated with type I APS. The presence of autoantibodies has been well documented and the nature of the involved autoantigens is being resolved.

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BIOGRAPHICAL SKETCH

Yangxin Li was born in Changchun, China, on June 6, 1965. Yangxin's dream since she was a middle school student was to become a scientist like Madame Curie. She won the second award in a national mathematics competition when she was 15 years old. Yangxin was listed among the top 10 students in Ji-Lin province in the national examination for graduated high school students. In 1989, she received a Bachelor of Science degree in physical chemistry at the University of Science and Technology of China (sponsored by the Chinese Academy of Science) which recruits top students from all parts of China. She then continued to do research at this university. In 1991, she went to Sweden to pursue a Ph.D degree where she married Yao-hua Song. She came to America with her husband on November 25, 1991. In 1992, she entered the graduate program at the Department of Pathology and Laboratory Medicine, University of Florida. After completion of her doctoral program, Yangxin will continue research in the field of autoimmune diseases, particularly, alopecia areata.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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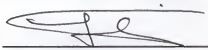

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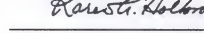

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